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ret/PTC Expression May Be Associated With Local Invasion of Thyroid Papillary Carcinoma

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Background and Objectives: The exact role of *ret*/PTC in the development of papillary carcinoma remains unclear. Expression of the *ret*/PTC oncogene was examined immunohistochemically to address its role in the progression of thyroid carcinomas.

Methods: Paraffin-embedded samples from 34 clinically evident thyroid papillary carcinomas and 19 occult papillary carcinomas were analyzed using an antibody raised against the *ret* tyrosine kinase domain.

Results: Expression of *ret*/PTC was demonstrated in 6/19 (32%) occult carcinomas. The frequency of expression of *ret*/PTC in clinically evident carcinomas in 16/34 (47%) was significantly higher than in normal tissues (0%) and follicular adenomas (1/14, 7%, $P < 0.01$). *ret*/PTC expression was observed more frequently in the peripheral areas of clinically evident carcinomas ($P < 0.01$). Although there was no correlation of *ret*/PTC expression with tumor size, lymph node metastasis, or distant metastasis, the incidence of *ret*/PTC expression in tumors with extrathyroidal invasion (13/19, 68%) was significantly higher than those without extrathyroidal invasion (3/15, 20%, $P < 0.01$). Local invasion was found in none of the occult carcinomas. The frequency of expression in occult carcinomas was significantly lower than in clinically evident carcinomas with extrathyroidal invasion ($P < 0.05$).

Conclusions: The *ret*/PTC oncogene may be involved in the local invasion of thyroid papillary carcinomas.

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KEY WORDS: ***ret*/PTC; *ret* proto-oncogene; thyroid cancer; invasion**

INTRODUCTION

The *ret* proto-oncogene maps to the long arm of chromosome 10 and encodes a membrane-associated tyrosine kinase receptor [1]. Its ligand is the glial-cell-line-derived neurotrophic factor [2]. Recent studies suggest that *ret* activation by mutations or rearrangements is associated with the development of medullary and papillary thyroid carcinomas. Germline mutations of the *ret* proto-oncogene have been identified in multiple endocrine neoplasia (MEN) types 2A, 2B, and familial medullary thyroid carcinoma (FMTC) [3–5]. Somatic mutations have also been demonstrated in sporadic medullary thyroid carcinoma [5,6]. Moreover, somatic rearrangements of the *ret* proto-oncogene have been uniquely detected in papillary thyroid carcinoma and in the rearranged forms of the *ret* gene designated *ret*/PTC oncogenes [7].

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Three *ret*/PTC oncogenes have been described in papillary thyroid carcinoma (*ret*/PTC1–3) [8–10]. In each case, rearrangements result in a fusion of the tyrosine kinase domain of the *ret* proto-oncogene to the 5′-terminal region of other genes that are constitutively expressed. *ret*/PTC1 and *ret*/PTC3 are thought to be formed by a paracentric inversion of the long arm of chromosome 10, fusing H4 and *ele*-1, respectively [8,9]. The fusion gene of *ret*/PTC2 contains the regulatory subunit RIα of the cAMP-dependent protein kinase A located on chromosome 17, and the rearrangement is thought to be created by a reciprocal translocation between chromosomes 10 and 17 [10]. The products of *ret*/PTC oncogenes have a constitutively activated tyrosine kinase function. Since *ret*/PTC is detected in papillary thyroid carcinoma, *ret*/PTC oncogenes appear to play a role in the pathogenesis of human papillary carcinoma. However, few studies of *ret*/PTC have examined correlations with clinical data [11,12]. We examined the relationship between clinical characteristics of patients with papillary carcinoma and *ret*/PTC expression by immunohistochemistry. Our data demonstrate an association between *ret*/PTC expression and local invasion in human papillary thyroid carcinoma.

MATERIALS AND METHODS

Tissues

Surgical specimens from 14 follicular thyroid adenomas, 34 clinically evident papillary thyroid carcinomas, and 4 medullary thyroid carcinomas were included in this study. In total, 37 specimens of adjacent normal thyroid tissues were also collected. Ten normal tissues were obtained from the cases with follicular adenoma. Twenty-six normal tissues were obtained from the cases with papillary carcinoma and one normal tissue was obtained from the case with medullary carcinoma. Nineteen specimens of occult papillary carcinoma were collected at autopsy. All tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin.

Immunohistochemistry

Paraffin-embedded sections (5 μm) were deparaffinized in xylene and graded ethanols, and then underwent a microwave antigen retrieval technique. The sections were then incubated with 0.3% hydrogen peroxide in methanol for 15 min to inactivate endogenous peroxidase. Nonspecific staining was blocked by preincubation with 2% bovine serum albumin for 5 min. The sections were subsequently incubated at 4°C overnight with the affinity-purified rabbit polyclonal antibody Ret(C-19) (Santa Cruz Biotechnology, Santa Cruz, CA) raised against a peptide corresponding to 19 amino acids at the carboxy terminus of the *ret* proto-oncogene tyrosine kinase. The primary antibody was diluted 1:100 in Tris-buffered saline. This antibody recognizes *ret*/PTC and *ret* oncogene products because both contain the *ret* proto-oncogene tyrosine kinase domain. Immunogenic peptide SC-167P (Santa Cruz Biotechnology) was also used along with Ret(C-19) for a preabsorption test to confirm the specific staining. Immunostaining was performed using the DAKO LSAB Kit (DAKO, Carpinteria, CA) with diaminobenzidine (DOJINDO, Kumamoto, Japan) as the chromogen. The sections were counterstained with hematoxylin. Normal rabbit immunoglobulin at the same protein concentration as Ret(C-19) served as a negative control antibody.

Evaluation of Immunohistochemical Staining

The percentage of cells stained with diaminobenzidine (i.e., the staining index) was determined by the microscopic examination of at least 100 cells. Tissues with a staining index greater than 30% were defined as positive for *ret*/PTC expression.

Statistical Analysis

Statistical analysis consisted of the chi-square test and unpaired or paired Student *t*-test. Values of $P < 0.05$ were considered significant.

RESULTS

ret Expression in Medullary Thyroid Carcinoma

A preliminary study of *ret* expression in four medullary thyroid carcinomas (one FMTC, one MEN type 2B, and two sporadic medullary thyroid carcinomas) was undertaken to confirm the validity of our method. Immunohistochemical staining with Ret(C-19) in the FMTC case, in which point mutation in exon 11 of the *ret* oncogene was previously demonstrated by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP), revealed diffuse cytoplasmic staining of tumor cells and no staining of normal thyroid follicular cells. There was no staining of tumor cells with the normal rabbit immunoglobulin control. Expression of the *ret* oncogene was also detected immunohistochemically in the other three medullary thyroid carcinomas.

ret/PTC Expression in Normal and Neoplastic Thyroid Tissues

The expression of *ret*/PTC was not detected in any normal thyroid tissues. Most follicular adenomas were also negative for *ret*/PTC expression (Fig. 1A). Only 1 of 14 follicular adenomas (7%) showed positive staining, and this *ret*/PTC positive tumor was histologically an atypical adenoma. Six of 19 occult papillary carcinomas (32%) and 16 of 34 clinically evident papillary carcinomas (47%) showed specific cytoplasmic staining (Figs. 1B, 2). Nuclear staining was also observed in some papillary carcinomas. The preabsorption test by immunogenic peptide was conducted in clinically evident papillary carcinomas that showed positive staining and no

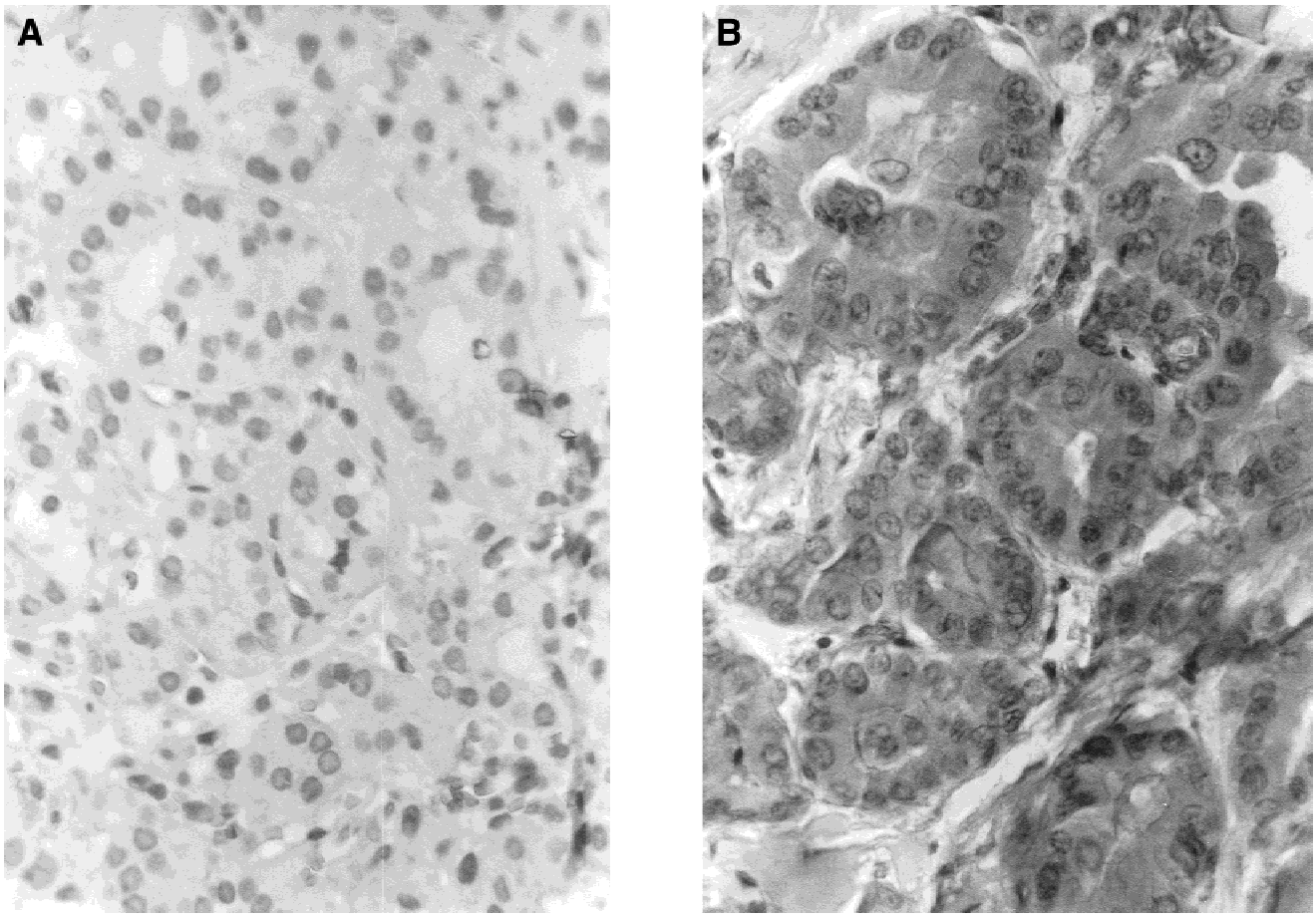

Fig. 1. *ret*/PTC expression in neoplastic thyroid tissues. **A:** Follicular thyroid adenoma (×200). No staining was observed in 93% of the cases with follicular adenoma, as shown in this figure. **B:** Papillary thyroid carcinoma (×200). Many cancer cells demonstrated cytoplasmic staining.

staining was observed after preabsorption. There was a significant difference in the frequency of *ret*/PTC expression between occult carcinomas and normal tissues ($P < 0.01$). The frequency of *ret*/PTC expression in clinically evident carcinomas was also significantly higher than in normal tissues and follicular adenomas ($P < 0.01$).

The staining index was different in different parts of clinically evident carcinomas (Figs. 3, 4). *ret*/PTC ex-

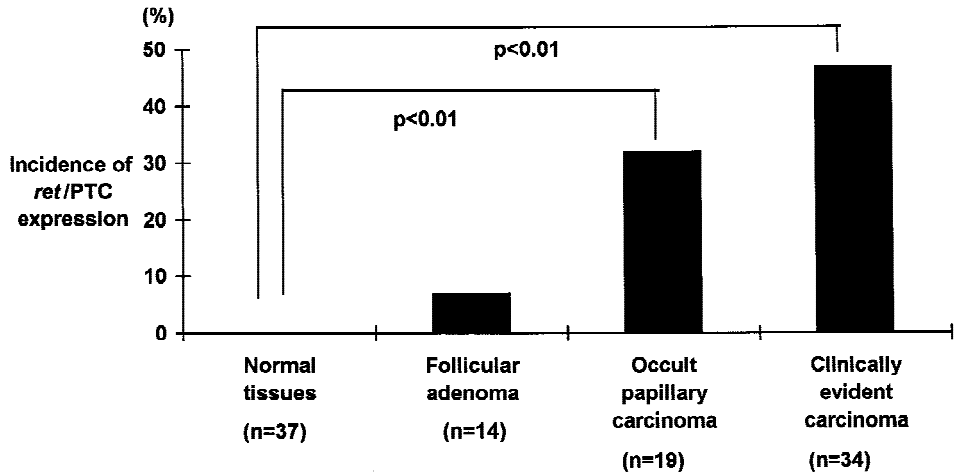

Fig. 2. Incidence of *ret*/PTC expression. Incidence of *ret*/PTC expression in occult papillary carcinomas (32%) was significantly higher than in normal tissues (0%) ($P < 0.01$). The incidence in clinically evident carcinomas (47%) was significantly higher than in normal tissues and follicular adenomas (7%) ($P < 0.01$).

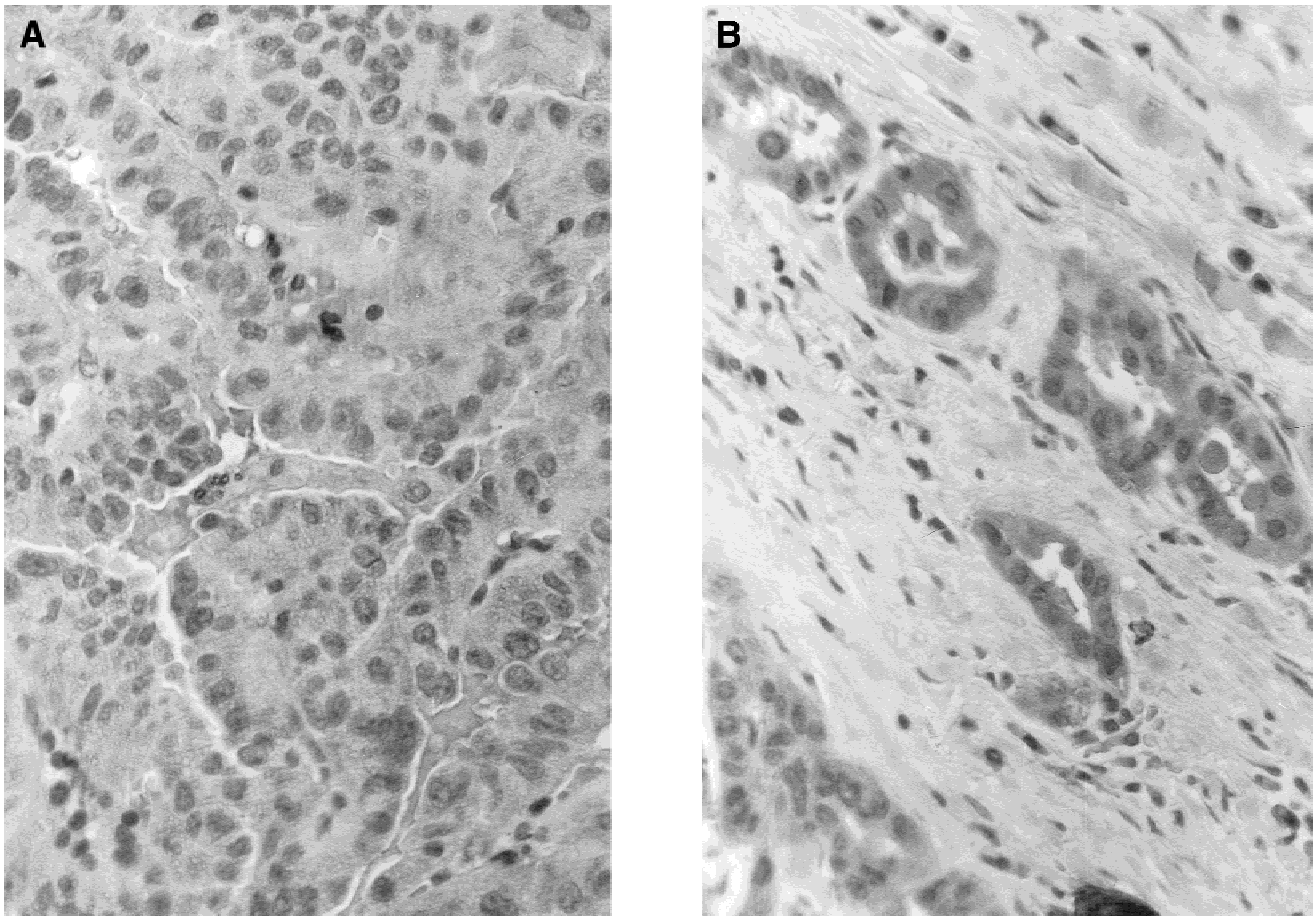

Fig. 3. *ret*/PTC expression in the central and peripheral areas of clinically evident papillary thyroid carcinoma. **(A)** Central area (×200). **(B)** Peripheral area of the same tumor as in A (×200). The percent of positively staining cells in the peripheral area was greater than in the central area, and the intensity of immunostaining in the peripheral area was also greater.

pression was predominant in peripheral areas compared with central areas ($P < 0.01$). Moreover, the intensity of staining in peripheral areas also seemed to be stronger. The staining index in different sections of occult carcinomas could not be examined because of the small tumor size (<8 mm).

Correlation of *ret*/PTC Expression With Clinical Data

ret/PTC expression in clinically evident carcinomas was examined in relation to important clinical data (Table I). There was no difference in patient gender or age between the *ret*/PTC positive and negative cases. The incidence of *ret*/PTC expression in cases with extrathyroidal invasion was higher than those without invasion (Fig. 5), although there was no correlation with tumor size, lymph node metastasis, or distant metastasis. In clinically evident papillary carcinomas, 13 of 19 (68%) tumors that infiltrated extrathyroidal tissues demonstrated *ret*/PTC expression compared with 3 of 15 (20%) tumors without extrathyroidal invasion ($P < 0.01$). All

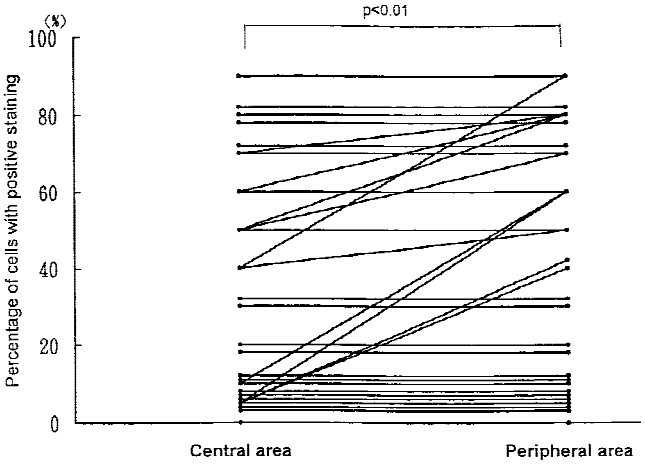

Fig. 4. Staining index of *ret*/PTC protein in the central and peripheral areas of clinically evident papillary thyroid carcinomas. The percentages of cells with positive staining in central and peripheral areas in each case were joined by a line when plotted on the graph. Staining index in the peripheral area was significantly higher than in the central area according to the paired Student's *t* test ($P < 0.01$).

TABLE I. *ret*/PTC Expression and Clinical Parameters

	ret/PTC expression	
	(+)	(−)
Number of cases	16	18
Age at diagnosis (yr)	55 ± 14	52 ± 13
Male:female	5:11	2:16
Tumor size (mm)	26 ± 19	21 ± 15
Lymph node metastasis	75%	83%
Distant metastasis	6%	11%

(mean ± Standard Deviation)

occult carcinomas remained within the thyroid capsule, and the frequency (32%, 6/19) of *ret*/PTC expression in occult carcinomas was significantly lower than that in clinically evident carcinomas with extrathyroidal invasion ($P < 0.05$). There was no statistically significant difference ($P = 0.36$) between the incidence of expression in occult carcinoma and that in noninvasive clinically evident carcinoma.

DISCUSSION

A rearrangement of the *ret* oncogene *ret*/PTC has been detected in papillary thyroid carcinoma and appears to constitute a specific genetic event related to the pathogenesis of human papillary carcinoma [13,14]. However, the exact role of *ret*/PTC in the development of papillary carcinoma remains unclear. Therefore, we studied *ret*/PTC expression by immunohistochemistry and examined the relationship between *ret*/PTC expression and clinical features of papillary carcinomas.

Recently, reverse transcription (RT)-PCR and immunohistochemical analysis have been used to study the *ret*/PTC rearrangements because of high sensitivity. Viglietto et al. [15] have reported a striking correspondence between results obtained with immunohistochemistry and RT-PCR on paraffin-embedded samples. Therefore, we employed the immunohistochemical method in this study to examine *ret*/PTC expression in archival paraffin-embedded tissues. The antibody used in this study was the same as used by Jhiang et al. [16], who determined the antibody specificity by the presence of staining in the thyroid parafollicular cells known to express the *ret* proto-oncogene. There existed the possibility that the antibody specificity varied within the batch. Therefore, we studied four tissues of papillary carcinoma by antibodies with two different lots. The results obtained by using these antibodies were identical. Moreover, the specificity of the antibody in this study was confirmed by preabsorption of the immunogenic peptide in papillary carcinomas.

The frequency of *ret*/PTC expression in our study was higher than in other studies [17,18]. However, the high frequency in our study could not be compared unconditionally with other studies because it might be attributable to the criterion of positive immunohistochemical staining. There were no normal thyroid tissues with a staining index greater than 20%. Therefore, we established 30% of positive cells in the whole area as the cutoff value in order to distinguish the positive staining from nonspecific staining. Some cases displayed a higher percentage of positive cells in the peripheral area than in the central area of the tumor. In such cases, the percent in the central area was regarded as representative of the tissue because the peripheral area was limited to comparison with the central area.

As the *ret* and *ret*/PTC oncogenes contain the same carboxy terminal tyrosine kinase domain, the antibody specific for the *ret* tyrosine kinase domain used in this study recognizes both *ret* and *ret*/PTC products. We conducted a preliminary study of *ret* expression in medullary carcinomas to examine the validity of our method. Nakamura et al. [19] observed the *ret* product in the cytoplasm of medullary carcinomas. Our examination of a FMTC in which *ret* activation had been previously demonstrated by PCR-SSCP demonstrated cytoplasmic staining restricted to neoplastic tissue. Activation of *ret* has been reported in 93–100% of MEN type 2B tumors and in 30–40% of sporadic medullary carcinomas [5,6]. We also detected *ret* expression in an MEN type 2B tumor and two sporadic medullary carcinomas in our preliminary study. Therefore, the validity of our immunohistochemical method was suggested from these results. In our study, *ret*/PTC expression was observed in one atypical adenoma, in which 80% of cells showed positive staining and positive cells were distributed uniformally. Although *ret*/PTC rearrangements have been found to be restricted to papillary carcinoma in other studies [1,13], Ishizaka et al. [20] demonstrated *ret*/PTC transcripts in 4 of 19 follicular adenomas. *ret*/PTC rearrangements seemed to occur not only in papillary carcinoma but also in some benign nodules.

Our finding that the cytoplasm of the tumor cell was stained immunohistochemically was compatible with

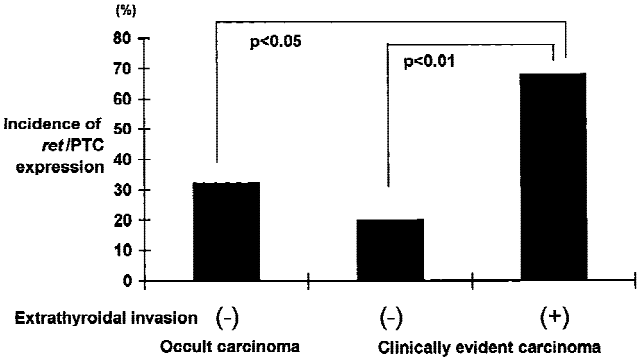

Fig. 5. Extrathyroidal invasion and *ret*/PTC expression. The incidence of *ret*/PTC expression in clinically evident papillary thyroid carcinomas with extrathyroidal invasion was significantly higher than in carcinomas without extrathyroidal invasion.

other studies [15,16], though the demonstration of differences in immunoreactivity of different parts of the tumor has not been previously reported. The important finding in our study was that the percent of cells with *ret*/PTC expression in peripheral areas was significantly higher than in central areas of the tumor. Moreover, the intensity of staining in peripheral areas appeared to be stronger than in central areas. This suggests that expression of *ret*/PTC may be related to the invasion of papillary carcinomas.

The clinical significance of *ret*/PTC remains unclear as few studies of the relationship between *ret*/PTC and clinical findings were reported. Jhiang et al. [11] reported that *ret*/PTC activation was higher in tumors from patients with distant metastases (2/4, 50%) than in tumors without metastases (4/32, 12%). Sugg et al. [12] reported that two of three patients who had papillary carcinomas containing *ret*/PTC rearrangements presented with lymph node metastasis. However, we did not find any correlation of *ret*/PTC expression with age, gender, tumor size, distant metastasis, or lymph node metastasis.

Two studies are relevant to the issue of local invasion. Di Renzo et al. [21] have reported that all three papillary carcinomas examined in which *ret*/PTC rearrangements were detected demonstrated extracapsular infiltration. Furthermore, another study reported that transgenic mice with thyroid-targeted expression of the *ret*/PTC1 oncogene developed thyroid carcinomas with considerable similarity to human papillary carcinoma, and all carcinomas revealed intrathyroidal and periglandular invasion without evidence of local or distant metastasis [16]. In our study, *ret*/PTC expression in both occult carcinomas and clinically evident carcinomas without extrathyroidal invasion was significantly ($P < 0.05$) less frequent than in carcinomas with extrathyroidal invasion. These results suggest that papillary carcinomas with *ret*/PTC expression may be more likely to invade locally. Examination of *ret*/PTC expression may be useful as an additional indicator of aggressive behavior in papillary thyroid carcinoma.

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COMMENTARY

Miki et al. have described their experience with *ret*/PTC expression in patients with papillary cancer of the thyroid. They have made efforts to correlate the *ret* expression and the local aggressiveness of thyroid cancer. A long-standing thyroid cancer will develop local extension with extrathyroidal spread and locally aggressive behavior. Whether it is the duration of the thyroid cancer, age of the patient, or the expression of *ret* that designs the aggressiveness of thyroid cancer remains unclear. A specific histological variety of poorly differentiated thyroid cancer often presents with a locally aggressive nature. *ret* mutation is very well described in patients with medullary thyroid cancer. As a matter of fact, this is the only tumor marker that has shown major clini-

cal application in early diagnosis of medullary carcinoma of thyroid in family members of patients with medullary thyroid cancer. Among the tremendous explosion of molecular biology today with substantial literature, *ret* proto-oncogene represents a unique contribution to the recent literature in the clinical application of early diagnosis of medullary carcinoma of thyroid. The development of cancer is a unique balance between the oncogenes and the tumor suppressor genes. p53, a well-known tumor suppressor gene, is studied in greater detail in many recent publications, especially for poorly differentiated and anaplastic carcinoma of the thyroid. There is a high expression of p53 in patients with anaplastic thyroid cancer. The extrathyroidal extension and locally aggressive thyroid cancer have a high incidence of local recurrence due to inadequacy of total resection of the tumor. Overall survival in this group of patients drops considerably compared with those without extrathyroidal extension.

Various tumor markers are being studied recently in the oncogenesis of thyroid cancer starting from normal follicular cells to the development of differentiated and undifferentiated thyroid cancer. Even with this explosion of molecular biology, the clinical applicability needs to be studied further. In view of this, even though I would like to congratulate the authors for their interesting work, I would like to caution the readers about the applicability of this phase I study. It will be important for the authors to continue their work and study a larger number of patients with differentiated and undifferentiated thyroid cancer and its effect on local control and overall survival results.

It is important to compare the results of immunohistochemistry with RT-PCR studies. It is interesting that the authors found no correlation with age, which appears to be the most important clinical prognostic factor in patients with thyroid cancer. It would be important to study this prospectively in fresh tissues rather than the archival material. Various tumor markers such as EGF receptor telomerase have been studied with contradictory results. In view of this, the clinical applicability of these markers needs to be studied further before making any definitive judgment.

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